

# The Cytoplasmic Inclusions of the Germ-Cells.

## PART VII. THE MODERN TECHNIQUE OF CYTOLOGY.<sup>1</sup>

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## 1. INTRODUCTION.

IN this paper I have collected some notes on technique, with especial attention to various methods used for demonstrating and distinguishing between protoplasmic and deutoplasmic bodies. I have also added directions and a plan for research on the lines introduced into this country by myself.

In a previous paper published in the 'Journal of the Royal Microscopical Society' (13) I have gone into certain other aspects of these questions, and this paper is meant to supplement what I have written before. The application of modern technique to experimental zoology and physiology will, I have little doubt, very shortly provide us with a wealth of facts with regard to the life of the cell and the development of the organism.

Where necessary I have made due acknowledgment to other workers, such as Murray, Cramer, Cowdry, Bensley, Kull, Hirschler, Regaud, Champy, Cajal, Golgi, etc.

Formulæ of fixatives such as Bouin, Flemming, Altmann, Champy, Gilson-Petrunkewitsch, Carnoy, etc., will be found in books on microscopical technique, and are not given here. This paper is written with the supposition that the reader is

in possession of a late edition of Bolles Lee (3) or some other such admirable book. Complete directions for every new method in this paper are given carefully, and full bibliographies will be found in the other parts of this series of papers.

## 2. The Cell Elements Classified.

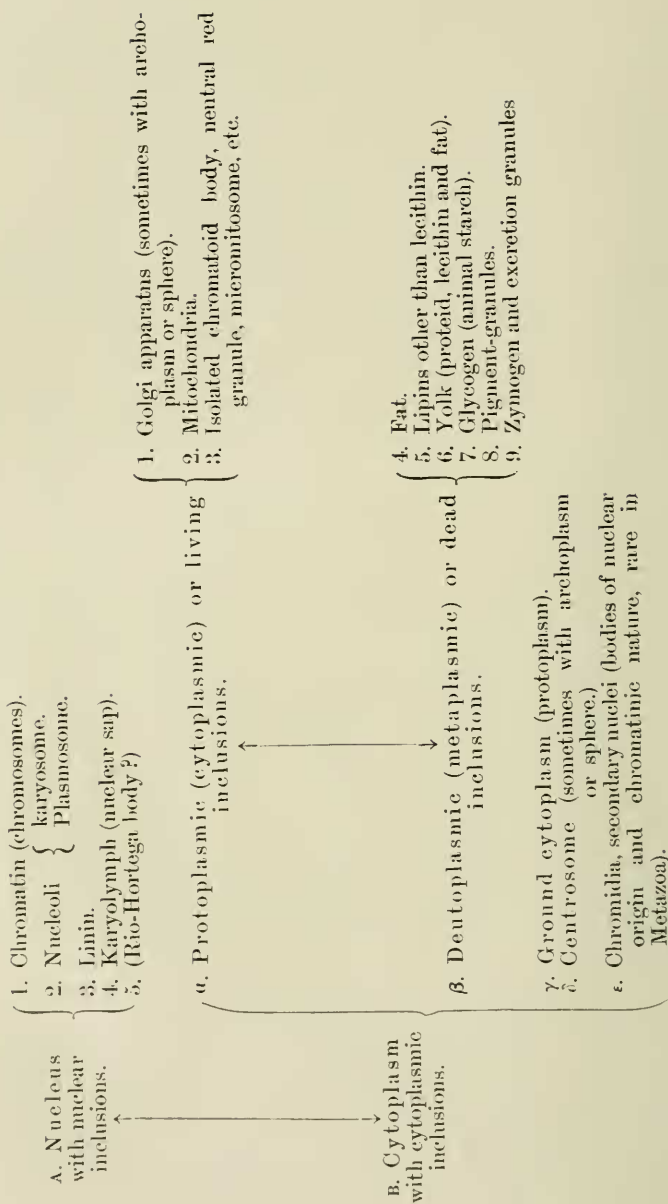
In the scheme on p. 270 I have given a tentative classification of the cell elements. The nucleus contains four well-known categories of substances or materials, and in addition in some cells it can be shown (Rio Hortega, 29) that an enigmatic granule or rod which is argentophile is seen to lie in or near the nucleolus. I have called this rod the "Rio-Hortega Body" after its discoverer, who is one of Cajal's pupils.

Between the words nucleus and cytoplasm I have drawn a double arrow to denote the interaction which goes on between these bodies. The cytoplasm is known to contain two general categories of bodies—metaplasmic or dead storage, excretion or secretion products, which also fall under the term "deutoplasmic," especially if they are nutrient, and protoplasmic bodies (active centres of vital changes), engaged in processes of cell metabolism. Between these two groups I have drawn a double arrow to denote exchange.

The deutoplasmic inclusions are especially fat, yolk, glycogen and "lipoid" vacuoles, while excretion granules and pigment are possibly different and are truly metaplasmic, but for the present it is somewhat difficult to separate out the dead cell inclusions into distinct classes. Pigment for instance may be associated with mitochondria and possibly also with Golgi elements.

With reference to the word "dead" and "living" as used in this classification, it should be pointed out that both mitochondria and Golgi elements are centres of cytoplasmic activity, and can be seen to grow and divide. Yolk-discs and fat-droplets are classified as dead, because they do not divide, and are not centres of cytoplasmic activity in the same sense or way as the mitochondria or Golgi elements.

## Classification of Cell Elements.





It is certain now that both protoplasmic and deutoplasmic inclusions are closely inter-related. Murray (24) and Dubreil (11) both describe the metamorphosis of mitochondria (living) into fat-globules (dead). It is also true that mitochondrial, zymogen, pigment and lipin granules or vacuoles are closely connected with one another. This serves to warn us that such a classificatory scheme as I have given here must not and cannot be regarded as rigid. That such a classification is useful I believe that there is no doubt, and it gives one some idea of the complexity of the cell.

Under the section of Cytoplasm I have given the ground protoplasm or cytoplasm and the centrosome a special heading by themselves, as is justified by their importance.

A classification of this sort can be the only basis of the cellular side of research in embryology, because we now know that the nucleus and ground cytoplasm are only two of the many bodies and substances which form the cell. The development and life of the organism are comprised by the behaviour and changes which go on in the complex system outlined in my classification. Attention is drawn to the tables which I have published elsewhere, and which are aimed at enabling the observer to identify any special cell-body by its fixing and staining reactions (13).

### 3. Are the Mitochondria and Golgi Elements Artefacts Produced by Certain Techniques?

Within the last few years the work of Regaud, Champy, Fauré-Fremiet, Meves, Held, Hirschler, Weigl, Nusbaum-Hilarowitz and Cowdry abroad, and of Murray and myself in this country, has focussed a good deal of attention on the mitochondria. More recently also I have entered into the question of the Golgi apparatus in zoological material, and Weigl, Cajal and Hirschler, too, have written papers on the Golgi apparatus in eggs. Naturally this attention has also been partly constituted by a scepticism as to the facts which I have lately published under the title of "The Cytoplasmic

Inclusions of the Germ-cells." The sceptics are almost always found to be ignorant workers who are amateur cytologists and microscopists, interested mainly in diatoms, and the mechanism and theory of microscopes. To these I commend the following facts, which even a cursory study of the international literature will confirm.

(1) The mitochondria can be seen and studied under the microscope, in freshly teased live animal-cells, or in the hairlets of the roots of plants, etc. (12, 15, 20).

(2) The Golgi apparatus of many sperm-cells is quite easily seen under the same circumstances.

(3) Intra-vitam stains tinge the same bodies, in the same location, in live untreated cells.

(4) Properly fixed and stained cells show the same bodies, the same size and shape, in the same location as in paragraphs (1), (2) and (3).

(5) Fixing and staining methods reveal such bodies in all stages of the germ-cell cycle in a variety of animals and plant-cells.

(6) The presence of a Golgi apparatus and mitochondria in animal-cells is now widely accepted by trained workers.

#### 4. Lipoids, Lipins, Phosphatides, Phospholipins or Substances in the Cell Allied to Fats.

Fats are soluble in acetone, xylol, chloroform and ether especially. It is found, however, that strong alcohol is not without effect on animal fat-globules. Now besides the true neutral fats, such as palmitin, stearin, and the unsaturated olein, it has been ascertained that there are other "fatty" substances in all cells, which are somewhat allied to the fats in their chemical nature. Such substances, which are broadly like the fats in their solubility reactions, have been called "lipoids," and are especially soluble in alcohol. Now it has been shown by the excellent worker Maclean (21), that ordinary fat solvents such as ether and chloroform, and also alcohol, extract a variety of different "lipoids" from the cell, ranging from cholesterol to pigments, and that while such

substances agree generally in their solubility reactions, they may be otherwise unrelated. Leathes (19), in 1910, suggested that the word "lipin" be used for Thudichum's so-called "amidolipotids," which may only be decomposition products. In order to clear up the confusion with regard to these peculiar "lipoid" substances, Maclean has suggested that the term "lipin" be used only for those substances of a fat-like nature, which contain in their molecule either nitrogen or nitrogen and phosphorus, and which yield on hydrolysis fatty acids or derivatives of fatty acids.

In the following section I intend to use the word "lipoid" in Overton's sense, i. e. to mean all those substances which dissolve in organic solvents, such as chloroform, alcohol, and ether.

The lipins comprise two special groups of substances—the phosphatides and the cerebrosides; the term "phospholipin" has been suggested instead of phosphatide. Phosphatides are fat-like bodies containing in their molecule nitrogen and phosphorus, while the cerebrosides contain nitrogen but no phosphorus. Acetone will not dissolve lipins but will dissolve fats. Biochemists extract lipins from dried tissues by means of ether and alcohol and precipitate the bodies from the solvent by means of acetone. Certain "lipins" at least form insoluble compounds with chrome mercury and platinum salts. "Lipoids" (e. g. cholesterol, lecithin, fat, cerebrin, etc.) form a special part of almost all cell-organs, as seems to be indicated by fixing tests, and so far as we know such substances are always intimately associated with protoplasm. From this we arrive at a definite conclusion as to the importance of work on "lipoids"; it is this: fixatives containing alcohol, chloroform and acetic acid especially either completely dissolve away "lipoids" of all kinds, or distort and alter their morphological arrangement in the cell or cell organs; while those fixatives which in themselves are not injurious, but which do not cause the lipoids to become insoluble in the dehydrating and clearing reagents, are not indicated for the best cytological work.

### 5. Yolk-Discs and Spheres.

Nearly all, if not all eggs contain yolk-bodies of some sort. In the majority of cases such yolk-bodies are spherical or discoidal, and are constituted by lecithin, free fat, and a coagulable protein which has been called vitellin (27). According to the amount of each of these three categories of substances, the yolk-sphere or disc will act in a varying manner under a given technique. In osmic acid the sphere may or may not go black, and I think this depends on the presence or absence of unsaturated free fat; in other cases it may or may not stain black or violet in iron-hæmatoxylin or Benda (3) respectively, and this apparently depends on the quantity of the protein contained in the disc.

Yolk-discs or spheres form most of the nutrient substance in eggs, and no two different animals have yolk-granules of the same chemical constitution. In birds the yolk-discs go black almost immediately in osmic acid, while in the frog and snail they do not do so. I do not think the yolk-disc of the frog is phyletically a different structure from that of a bird or a snail, and probably the yolk-disc of any animal will be found to contain the three categories of substances—fat, lecithin, and proteid—in stable equilibrium. Since true yolk-spheres contain either lecithin or proteid it is not difficult to discriminate between them and fat-spheres. Fat-spheres or vacuoles, yolk-spheres, and true lipin spheres, such as occur occasionally in fatty tissue and liver, are nevertheless liable to become confused with one another. (See section on turpentine extraction, p. 287.)

### 6. The Problem of Fixation.

Older cytologists were satisfied if their preparations showed the nuclei and cell walls unshrunk and clearly marked. Nowadays, however, it can be recognised that the problem of fixation is complicated. Besides the coagulable proteins, the cell contains a number of substances, many of which are so

easily altered or dissolved that special precautions must be observed in fixation. The materials in the cell which collectively fall under the term "lipoid" are rapidly destroyed by alcohol, acetic acid, chloroform, and are altered by certain other fluids. Not only this, but it is necessary to mix one's reagents in such a way as to avoid distortion; it should be noted that the problem of fixation is not constituted only by the questions as to how various chemical reagents affect any cell substance, but also by the question as to how such a treated substance will resist the subsequent dehydration and embedding. For instance formol fixation does not injure fat in cells, but it will not keep alcohol and xylol or chloroform from subsequently dissolving it out of the tissue; osmium tetroxide also does not injure fat in cells, but in addition it keeps the alcohol and xylol or chloroform from dissolving it away subsequently.

From the cytological and protozoological points of view the problem of fixation may be said to be constituted by two difficulties: (A) Avoidance of fluids which dissolve out or distort cell elements; (B) avoidance of fluids which enable or cause cell materials to be soluble in the dehydrating and clearing agents. As an example I will take the technique widely used by protozoologists, i. e. that of Schaudinn's fluid or Bouin's fluid and iron-alum hæmatoxylin. Schaudinn's fluid is a typical alcohol-acetic acid and corrosive sublimate fixative; the application of it to the cell possibly results as follows: Some of the proteids are coagulated, but part at least of such coagulated proteids are water-soluble. The acetic acid and alcohol rake all lipins out of the cell, while other fatty bodies are either dissolved away, or left in a state which allows them to be dissolved away in subsequent dehydration and clearing. Then the cells are stained in watery alum hæmatoxylin, which dissolves out those water-soluble proteid salts. Some workers have partly done away with the latter difficulty by using an alcoholic hæmatoxylin. Bouin's fluid is possibly less actively destructive than Schaudinn's but it is not indicated for cytological protistology. A

paper bearing on this subject has recently been published by Dr. Ward Cutler, who has especially studied *Ditrichomonas*, a remarkable flagellate from the gut of a termite (10). In certain *Trichomonads* are found a row of coarse protoplasmic granules, whose correct identity is difficult to recognise. Cutler calls them "metabolic granules," and he shows that in most ways chrome osmium fixation gives the truest picture of the fixed organism. There is no other branch of zoology quite like protistology, for in the latter the most perfect technique is needed and is rarely found. Most protozoologists publish figures of their bad preparations, with the mistaken opinion that what they draw is something like the organism *intra-vitam*.

#### 7. Ten Common Reagents Used as Fixatives.

These reagents are as follows :

- |      |                                      |
|------|--------------------------------------|
|      | 1. Osmium tetroxide in water.        |
|      | 2. Formaldehyde gas in water.        |
| A. { | 3. Chromium trioxide in water.       |
|      | 4. Bichromate of potassium in water. |
|      | 5. Platinum chloride in water.       |
|      | 6. Mercury bichloride in water.      |
|      | 7. Nitric acid.                      |
| B. { | 8. Picric acid.                      |
|      | 9. Alcohol.                          |
|      | 10. Acetic acid.                     |

Chloroform and urea are also used. In the group marked A are arranged the valuable reagents, in B the less valuable or destructive ones. Good fixatives can be made from the substances in group A without using any of the reagents in group B. The latter contains the reagents useful for chromosome work, the former reagents useful for fixing the cytoplasm and "resting" nucleus.

From group A have been made the following mixtures: Altmann, Champy, and Flemming and Hermann-without-acetic acid; these are among the best mixtures known. Then



there are formalin (5 per cent. to 10 per cent.), Regaud, formol-Müller and formol-Flemming, which are so good for mammals. Good general micro-anatomical fixatives from group B are Zenker, Bouin, Gilson-Petrunkewitsch and Schaudinn. These all destroy much of the cell-contents, and give an incorrect picture of the cell, excepting chromatinic structures, for which they are indicated.

### 8. Penetration of Fixatives.

Penetration is the power that a fixing fluid has of passing inwards through the cells forming a tissue. The best penetrators are alcohol, acetic acid and formalin. Less so bichromate of potash and corrosive sublimate. Picric acid also penetrates well. Of the active penetrators, formalin alone is non-injurious to various lipin and fatty substances; acetic acid and alcohol are among the most harmful substances which can be applied to the cell. Osmic acid is a very poor penetrator.

Very few (if any), fixatives penetrate a large piece of tissue quite evenly, and the outside of an organ is almost always fixed differently from the inner regions, because as the reagents soak inwards, some are altered by chemical action, and so the qualities of the fluid become altered in relation to the tissue. In some ways (especially to the cytologist) this may be an advantage, for at certain regions of the tissue the most suitable gravity or strength of the fluid may be arrived at automatically, and so in these regions the cells will be perfectly fixed. It is doubtful how far in fixing mixtures the separate reagents keep together.

The problem of producing some fixing mixture which will penetrate chitin and cyst-walls, and at the same time will not destroy lipins and fats, is still unsolved. Fixatives like Carnoy and Petrunkewitsch are not suitable on account of their alcohol and acetic acid, while formalin does not penetrate sufficiently well for this purpose. (Steam fixation followed by strong chrome-formalin might be a possible method.)



### 9. General Note on the Use of Osmium Tetroxide ( $\text{OsO}_4$ ).

Osmium tetroxide solution, or osmic acid as it is often called, is the most valuable cytological reagent. Certain substances, such as the unsaturated fat, olein, have the power of reducing the  $\text{OsO}_4$ , or in other words of being oxidised by the osmic acid. Besides the fats olein, stearin and palmitin, which are almost always mixed in animal tissues, the zoologist is liable to meet lecithin in the form of yolk-discs. Such discs, as is well known, are never pure lecithin, but may contain, besides lecithin, a coagulable protein (vitellin), a free fat and cholesterol. The free fat may or may not always be present in yolk-discs, such as those of frog or snail eggs, but it is a fact that the discs in these eggs generally do not go quite black even when the eggs are soaked for fourteen days in the osmic solution. Mann (22) also has noticed that lecithin does not reduce osmic acid; when yolk-discs do go black in osmic acid, it probably means that, not the lecithin, but the associated free unsaturated fat, has reduced the osmium tetroxide solution. The ordinary cytologist, who may be more especially interested in the morphology and behaviour of certain cell-granules, only recognises fat, lecithin, glycogen or starch as they occur in granular form. It is known of course to most zoologists that the ground protoplasm of the cell contains a number of lipins which do not appear in prepared sections, but which have been detected by extraction or desiccation methods. When, however, such materials are present in such quantities as to form granulations, or to alter the form of the ground protoplasm, they come within the province of the cytologist.

A reaction well known to everyone is that in which the myelin sheath of medullated nerve goes black when treated fresh in osmic acid solution. Cramer, Feiss and Bullock (8) affirm that the myelin sheath of medullated nerve contains only such "lipoids" as phosphatides, cerebroside and cholesterol. The phosphatides comprise such substances as lecithin and cephalin, the cerebroside is represented

by cerebrin and the phospho-cerebrosides by protagon. It is obvious that there must be some substance in the nerve-sheath that reduces osmic acid, and this serves to warn us that not every substance which is turned black by osmic acid is unsaturated neutral fat. Kopsch's method for the Golgi apparatus ( $\text{OsO}_4$  for two weeks) is another such reaction in all probability, but whether the Golgi apparatus does contain olein we cannot say; what evidence we have points against this assumption.

It is clear that the number of animal substances which have the power of normally reducing osmium tetroxide solution is limited, but it is at the same time equally clear that osmic acid alone is not a specific test for fat. As has been pointed out before, the presence of either chromic acid or bichromate of potash with the osmic acid prevents the blackening of the nerve-sheath or of the Golgi apparatus, but not of animal fat. If a globule in a cell goes black after treatment in Flemming-without-acetic acid, or Altmann, one can safely assume that it is fat or contains fat. In addition the polariscope microscope will be found to help in distinguishing fats and true lipins (see p. 295), and also Cramer (7).

There is no doubt that some of the more capricious results obtained with osmic acid are due to neglect of proper precautions. Capsules and tubes used for the various osmic acid techniques should be scrupulously clean; the water used for making the osmic acid solution should be distilled and as free from dust and other impurities as possible; the solution of osmic acid should be kept in the dark; much discoloured crystals of osmic acid should be discarded, and very old solutions of the salt should not be used. If these precautions are taken it will not be found that  $\text{OsO}_4$  gives inconsistent results as certain observers have written.

#### 10. Osmium Tetroxide, Formalin, etc., and Some of their Reactions.

(A) Fat and Mitochondria Preservation.—Animal fat usually consists of a mixture of olein, palmitin and

stearin, and osmic acid appears to have the power of converting fat-vacuoles into some substance which is not immediately dissolved out by alcohol, xylol or chloroform. The general reaction of osmium tetroxide is to turn fat black. But this is not all; osmic acid has the power of converting lipins and lecithins into compounds which are insoluble in alcohol, xylol and chloroform. I conclude that this is so by direct experimentation on the granules, fatty or lipid, found in cells.

The important fact is to be noted that osmic acid is the only fixative which has the power of preserving fat, lecithin, and phosphatide (lipin [mitochondria]) at the same time. Cells containing fat, yolk and mitochondria, fixed in osmic acid and then passed through alcohol (dehydrating) and xylol or chloroform (clearing), are found not to have lost any part of their contents. What, however, do we find with formalin, platinum chloride, bichromate of potash and chromic acid? Fix such a cell in any of these reagents, and pass it afterwards through alcohol and xylol or chloroform, and the fat is removed, while the albumen and phosphatide (lipin, [mitochondria]) remain. Yolk-discs (lecithin) generally remain after this treatment, but are often shrunken. Formalin is particularly active in preserving mitochondria, possibly more so than the chrome salts. It must be noted that these reactions do not mean that formalin and the latter salts themselves destroy fat; it simply means that neither formalin nor the chrome salts are able to act upon fat in such a way as to prevent the latter's being dissolved out in alcohol and the clearing oil; but if one takes a piece of tissue and fixes it in formalin of from 5 per cent. to 10 per cent. or formol-bichromate (as of Regaud), and then treats small pieces of the tissue afterwards in  $\text{OsO}_4$  solution, or in chrome-osmium, the fat will be preserved, and will appear in the finished sections. This is an important point for workers on Vertebrate tissues. Thus Müller-formalin, Zenker-without-acetic acid, chromic acid and Zenker-formalin preserve mitochondria and not fat in the finished sections; chrome-osmium, platinum chloride-

osmium (Hermann), Kopsch and Altmann, preserve both fat and mitochondria.

(B) Certain fixatives (Ranvier, Kolossow, the old Flemming, etc.) contain alcohol or acetic acid mixed with osmic acid; such mixtures are illogical, because the latter tries to preserve what the former fluids distort or destroy. In certain cases it is found that acetic-osmic acid will dissolve away mitochondria and preserve fat; this is the reverse of what happens in the formol-chrome methods of Regaud or Müller, where mitochondria are preserved and fat extracted. Among invertebrates especially, the acetic acid and osmic fixatives will not preserve both fat and mitochondria, but in some vertebrate tissue the mitochondria are much more resistant.

(c) The reactions of osmic acid with the Golgi apparatus and the mitochondria are rather difficult properly to understand. When animal fat turns black with  $\text{OsO}_4$ , it has been supposed that the  $\text{OsO}_4$  is reduced either to  $\text{OsO}_2$ ,  $\text{OsO}$ , or to metallic osmium. Another account is that the  $\text{OsO}_4$  becomes hydrated to form  $\text{Os}(\text{OH})_3$ . Not claiming to sufficient knowledge of chemistry to enable him to criticise such explanations, the ordinary cytologist can but try to see how such explanations accord with practical cytological technique. What he does find is that a fat-globule not only turns black throughout in  $\text{OsO}_4$ , but that subsequent dehydration, clearing and embedding (if done carefully) do not cause the globule of fat to shrink in size. Obviously the fat must in some way be altered and made insoluble before use of the dehydratory and clearing agents. The explanation of this is difficult to formulate. One of my chemical friends has suggested that the osmium is brought down to a colloidal condition. I hope that this may be thoroughly examined by a chemist.

If a tissue is left for some time (one to two weeks) in 2 per cent. osmic acid, not only will the fat turn black, but the Golgi apparatus will go quite black, and in the cases examined by me the addition of chromic acid, bichromate of potash and platinum chloride to the  $\text{OsO}_4$ , while not preventing the fat from going black, does prevent the Golgi apparatus

from so doing. In other words,  $\text{PtCl}_4$ ,  $\text{CrO}_3$  and  $\text{K}_2\text{Cr}_2\text{O}_7$  inhibit the special osmic acid reaction for the Golgi apparatus. As an explanation of this, it might be assumed that the blackening of the Golgi apparatus by osmic acid is an oxidising process, but that the admixture of a more powerful oxidiser such as chromic acid to the osmic liquid prevents the latter from carrying out the reaction. Why, however, the Golgi apparatus should not go black under these circumstances, while fat does so, I cannot say. Nor can one say whether the substance of the Golgi apparatus which turns black with  $\text{OsO}_4$  is a true fat (olein). Apparently it is a lipin and not a fat.

#### 11. Bichromate of Potash or Chromic Acid with Osmic Acid, and Fats and Lipins.

Osmic acid alone may on rare occasions turn almost any cell element black. This largely depends on whether the osmic solution being used is old or freshly made, and often on certain factors more obscure. In fact the more one uses osmic acid, the more one is led to believe that its reactions are very difficult to understand. This must not be interpreted to mean that it is impossible to get reliable results by using osmic acid. It was mentioned before (12) that Flemming fluid without acetic acid does not blacken the Golgi apparatus in mollusc gonads, while osmic acid alone will. In the Marchi method to demonstrate degenerate nerves, parts of the nervous system containing regions of degeneration are fixed in Müller ( $\text{K}_2\text{Cr}_2\text{O}_7$ ); they are then treated in  $\text{K}_2\text{Cr}_2\text{O}_7$  and  $\text{OsO}_4$ , and it is found that the normal medullated sheaths will not go black in the  $\text{OsO}_4$ , while the diseased ones will. As is well known, osmic acid alone will turn the normal sheaths black. The treatment with the chrome-salt prevents this reaction taking place in all but the degenerate sheaths. In nerve degeneration it appears that glycerides of unsaturated fatty acids are formed apparently at the expense of the lipins which constitute the normal medullated nerve-sheath. It is this unsaturated non-phosphorised fat which, according to

Cramer, is responsible for the "Marchi reaction." For the zoologist the importance of this is very considerable; any substance in a tissue which goes black in Altmann, Hermann or Flemming-without-acetic is almost (though not quite) certain to be fat, or a glyceride of the unsaturated fatty acid such as olein.

The zoologist working on development and cytology in general is satisfied if he is able to identify any substance as "fat" or "oil," and distinguish it, if possible, from yolk-spheres or discs. Cramer considers that the sheath of the medullated nerve contains no unsaturated fat or fatty acid, but only lipoids such as lecithin, protagon and cholesterin, and he maintains that all turn black with osmic acid. Not being a skilled histo-chemist, I feel that it may be injudicious for me to say that I doubt Cramer's statement. Mann (22) states that lecithin (term used widely) does not become black with  $\text{OsO}_4$ , and I find that the yolk-discs of frog's and mollusc's eggs do not go black in osmic acid. I suggest that the substance in the sheath of medullated nerve which is blackened by  $\text{OsO}_4$  is not lecithin; it may be cholesterin or protagon—I feel unable to comment on whether these substances do turn black in  $\text{OsO}_4$ ; the only occasions yolk-discs immediately go black in osmic acid is when they contain associated unsaturated fats (Aves) or after Sjövall's method (13). In explanation of the "Marchi reaction" and the chrome-osmium blackening effect on fat and not on the Golgi apparatus it may be assumed that of the two oxidising reagents, the chrome salt is the most effective in the case of the Golgi apparatus and with lipoids generally, but does not oxidise the fat completely if at all.

## 12. Osmic Acid Vapour Fixation.

It is well known (3) that osmic vapour is very penetrating and toxic, and rapidly fixes smears and small pieces of tissue. My friend Dr. Cramer tells me that adrenalin granules in the suprarenal glands are best demonstrated by suspending a small organ (or a part of it) in a slightly wet iron gauze bag, over osmic acid in a glass-stoppered tube at  $37^\circ \text{C}$ . for one and a-half hours. By this method the vapour penetrates into the



tissue without the addition of any water. The main point to note is that substances in a tissue which might be dissolved out or altered by the water added to the  $\text{OsO}_4$  crystals are fixed in situ, and without the danger of alteration. This method should be of value to histologists and cytologists. Cramer directly transfers his fixed tissue to 50 per cent. alcohol and then up grades to absolute alcohol and mounts sections unstained. My three modifications of this method are as follows:

(A) Fix as above for one and a-half hours and then transfer to 2 per cent.  $\text{OsO}_4$  in water at  $37^\circ \text{C}$ . for one to several days. Then wash in water for several hours, dehydrate, embed and section. Mount unstained, or stain in acid fuchsin (Altmann) or iron-haematoxylin.

(B) Or fix as above for one and a-half hours and transfer to 1 per cent. chromic acid, or 3 per cent.  $\text{K}_2\text{Cr}_2\text{O}_7$  for several days. Wash several hours in running water and stain in acid fuchsin, or mount unstained.

(C) Tissues may also be fixed as above, and then transferred to Altmann's fluid, and subsequently stained in Altmann's fuchsin and picric acid. These methods should be useful to protozoologists. (See section on post-chroming.)

### 13. New Modifications of Kopsch's Method for the Golgi Apparatus, etc.

In my previous paper (13) I gave Kopsch's method. It consists of leaving small pieces of fresh tissue in 2 per cent.  $\text{OsO}_4$  for from one to two weeks at room-temperature, then washing, dehydrating, and embedding. Sections mounted without staining show the Golgi apparatus and the fat (sometimes yolk) black. Mitochondria rarely go black, generally yellowish. This unmodified method is quite good for Mollusca, Amphibia, and Mammals. It gives poor or often completely negative results for Arthropoda. Moreover, Kopsch's unmodified method generally causes bad shrinkage. The new methods are as follows:



A. Primary fixation in formalin (Sjövall) of a suitable strength (circa 5-10 per cent.), followed by slight washing in aq. dest.      Secondary treatment for several hours or one or two days in  $\text{OsO}_4$  of 2 per cent. Then wash in distilled water several hours and dehydrate.

B. Primary fixation in Mann's corrosive osmic (22) or corrosive alone for two or three hours, followed by aq. dest. as before.      Secondary treatment for from one to several days up to two weeks in  $\text{OsO}_4$  of 2 per cent. Wash as above.

To increase the power of method B, the secondary treatment may be carried out in thermostat at a temperature from 20° C. to 40° C. for one or two days.

Note also Kopsch-Altmann, mentioned in my previous paper (13).

#### 14. The Flemming-without-Acetic Acid and Iron-Hæmatoxylin Technique.

Flemming-without-acetic (F.W.A), Altmann or Champy, are chrome-osmium fixatives, which are admirable when followed by iron-hæmatoxylin or Altmann's acid fuchsin and picric acid. Since my most successful preparations have been procured with the F.W.A. iron-hæmatoxylin technique, it may be useful to other workers to give details of the process.

Small organs freshly dissected out in normal saline, or parts of organs cut with a sharp scalpel, not more than 5 mm. in diameter, are placed in about 15 c.cm. of one of the above-mentioned fixing fluids. A glass-covered capsule is the best vessel to use, and the material is left for at least twenty hours, and not longer than one week. I find about twenty-four hours gives a satisfactory fixation of most tissues. After fixation the liquid is poured away, and the material is washed for at least two hours, and not necessarily longer than five, in running tap-water. It is then passed through up-graded alcohols, beginning at 30 per cent., giving the material at least three hours in the strengths 30 per cent.,

50 per cent., and 70 per cent., and overnight in 90 per cent. The pieces of tissue are dehydrated two or three hours in two changes of absolute alcohol, and then transferred to a mixture of half absolute alcohol and half xylol for one quarter of an hour. They are left in pure xylol for another quarter of an hour, and then a mixture of cold wax and xylol is thrown into the capsule. The latter is transferred to the thermostat and left for half an hour, and then the liquid is poured away and pure melted wax is added. This is left for an hour, and the block is prepared and cooled in the ordinary way. Sections are cut from 4 to 8  $\mu$ , but I generally find 6  $\mu$  to be convenient. Sections are stuck on the slide with dilute glycerine and albumen water, and left overnight on the warm plate. The wax is melted off in xylol, and the sections are brought through alcohols to distilled water. They are mordanted from six to ten hours in 4 per cent. iron-alum, washed once or twice in distilled water, and transferred to hæmatoxylin of  $\frac{1}{2}$  per cent. for ten to fifteen hours. They are then washed in tap-water, differentiated in the iron-alum to the correct degree and washed again for five minutes, and then dehydrated and mounted; or they may be counterstained in orange G. For Altmann's acid fuchsin and picric acid stain, a previous fixation or a post chroming in some fluid containing bichromate of potassium gives the best results; Altmann's fluid is admirable. (See section on post-chroming.)

#### 15. Formalin and Iron-Hæmatoxylin.

For a microchemical test, and for clearly demonstrating the mitochondria, formalin and iron-hæmatoxylin are very good.

To 25 parts of formalin add 75 parts of distilled or tap-water (the latter is often more desirable). Neutralise the solution with magnesium carbonate. Leave pieces of tissue about the size of one's small finger-nail, in 20–30 c.cm. of the fluid for two days. Wash for half an hour each in 30 per cent. and 50 per cent. alcohol and leave for two hours in 70 per cent.; transfer to 90 per cent. and leave overnight. Finish deliy-

dration, clearing and embedding, and stain sections in iron-alum hæmatoxylin—alum three hours, hæmatoxylin from five to nine. In this connection see also Regaud's (28) fixation method (p. 289) which is admirable for mammalian tissues and gives splendid results when followed by iron-alum hæmatoxylin. After Regaud fixation, dehydration need not be carried out so rapidly.

#### 16. Post-Chroming (Mordantage).

To get a good Altmann stain and often to get any sort of satisfactory stain of mitochondria one must post-chrome, if the tissue has not been fixed in a mixture containing bichromate of potassium.

Material may be first of all fixed in formalin, corrosive sublimate, Zenker, chrome-osmium or such fixatives as those not containing alcohol, acetic acid and chloroform, and subsequently left for three to seven days in 3 per cent. potassium bichromate, then washed under tap overnight.

In addition smears or tissues may be fixed in osmic or formalin vapour for some time (one to two hours), and then left several days in the bichromate, washed overnight in running water, and then dehydrated, giving at least five hours to each strength; in some cases ten hours gives better results. The tissue may then be embedded, or smears may be brought down again to water and stained. Post-chroming is used in the methods of Benda and in the latest modifications of Altmann. The point to be noted here is that the well-known mitochondrial stains can thus be used even if tissue has not been first fixed in a special mitochondrial mixture.

Another post-chroming method, which mordants tissues prior to staining, is to soak the material in a mixture of pyroligneous acid (1 part) and chromic acid of 1 per cent. (2 parts) for one to several days. Then transfer the material or slides to 3 per cent.  $K_2Cr_2O_7$  for from three days to a week.

#### 17. Osmicated Fat, etc., and Turpentine.

Osmic acid fixatives have the disadvantage, in working with materials containing much fat or yolk, of blackening

and obscuring the sections. Other cell elements may be obliterated by a cloud of black or brown granules of fat. Since many of the modern techniques for mitochondria and Golgi apparatus consist partly in the use of osmic fixatives, this is a serious difficulty. It can be got over quite easily, however, as my friend Dr. J. A. Murray has pointed out to me, by soaking the sections on slide in turpentine oil, which has been left in sunlight (Bolles Lee, 3). Sections are prepared by any special osmic or osmic-chrome method, the wax is melted off them in xylol, and they are then transferred to a jar of turpentine. In some cases the black osmicated fat-globules come away in streams almost immediately; in other cases the process must be carried on overnight. When this method is tried on yolk-grains such as those of mollusc gonads, the brown or black colour is removed after a few hours and the yellowish disc or sphere remains. After treatment in turpentine the mitochondria can be stained in iron-alum hæmatoxylin.

I find that the Golgi apparatus in the gut-cells of the rat and of the newt (Molge) resists this treatment and remains black, while the fat- and yolk-granules are extracted and decolorised respectively. This method is important, and should be carefully noted by those who have found the diffuse blackening of cytoplasmic granules in osmic fixation to be a disadvantage. It can also be used to help in discrimination between Golgi granules and fat or yolk.

#### 18. The Acid Fuchsin Methyl-Green Modifications of Altmann's Mitochondrial Stain.

Altmann's acid fuchsin and picric acid stain has in my hands given satisfactory results, and I have not noticed that it faded much. By it mitochondria are stained reddish to reddish-purple according to the sample of fuchsin used, while the cytoplasmic background is yellowish with a reddish tinge. The nucleus is reddish. Bensley (2) substitutes methyl green for the picric acid, and according to Cowdry (6) this modification of Altmann's method is one of the most specific stains

for mitochondria. The latest modification used by Cowdry (6) is rather complicated and long; but provided a good specimen of acid fuchsin is obtainable the result is excellent and the stain can easily be learnt.

I regard Altmann's, Kull's or the Bensley-Cowdry stain as a natural and useful alternative to iron-hæmatoxylin. Regaud's mixture gives excellent results followed by iron-hæmatoxylin, so both methods can be used for sections cut from the same block. The same applies to Harry Kull's method given below (p. 290).

(A) Cowdry's Modification of the Altmann-Bensley Stain (Bensley-Cowdry).—(1) Fixation.—Regaud's mixture, 4 parts of 3 per cent.  $K_2Cr_2O_7$  and 1 part commercial formalin (neutralised by saturation with magnesium carbonate). Change mixture every day for four days; mordant for eight days in 3 per cent.  $K_2Cr_2O_7$ , changing every second day.

(2) Wash in running water overnight.

(3) Dehydrate, clear and embed in wax; cut  $4\mu$ .

Staining.—(1) Pass slides down through toluol, absolute alcohol, 95 per cent., etc., to water.

(2) Dip in 1 per cent. potass. permanganate thirty seconds or so.

(3) Rinse in 5 per cent. oxalic acid thirty seconds. (Note: Steps (2) and (3) may usually be dispensed with.)

(4) Rinse in distilled water about one minute or longer.

(5) Stain in Altmann's anilin fuchsin as follows: Make a saturated solution of anilin oil in distilled water by shaking the two together. Filter, and add 10 gm. of acid fuchsin to 100 c.c. of the filtrate. The stain is ready in twenty-four hours, but goes bad in a month. Dry around sections with duster, cover sections in fuchsin, and heat over lamp till anilin fumes steam off; allow to cool; let stain remain on about six minutes, and return former to bottle.

(6) Blot; rinse in distilled water as long as is experimentally found convenient.

(7) Pipette a little 1 per cent. methyl green over sections

for some five seconds or so as indicated. This is the crucial point of the method.

(8) Drain; transfer to 95 per cent. alcohol for a second or two. Absolute, toluol, balsam.

The next method, that of Harry Kull, will be found easier and quicker in some ways ('Anat. Anz.,' xlv, 1913).

(B) New Modification of Altmann's Method (Kull's Method, or Champy-Kull).—The following method, while being generally useful, will be found very convenient for work on Invertebrata. It gives results intermediate between that of Benda and Altmann, but is shorter and possibly better than the method of Benda. It will be found very useful for embryological research, and probably also for protozoology: Fix in Champy for twenty-four hours. Champy's fluid is as follows: Seven parts 1 per cent. chromic acid, 7 parts 3 per cent. bichromate of potash, and 4 parts of 2 per cent.  $\text{OsO}_4$ . Pieces to be fixed must be small. After fixation wash half-an-hour in aq. dest., and then transfer to a mixture of 1 part acid. acet. pyrolignosum rect., and 2 parts 1 per cent. chromic acid for twenty-hours. Wash half an hour in aq. dest. and transfer to a 3 per cent. solution of potassium bichromate for three days. Wash under tap for twenty-four hours; pass through up-graded alcohols to xylol; embed in paraffin wax (or celloidin method if desired). Section 4 or 5  $\mu$ .

Stain as follows: (1) Stain in Altmann's acid fuchsin aniline oil mixture (20 grm. of acid fuchsin in 100 c.c. of aniline oil water) and heat till steaming.

(2) Set slide aside to cool (this is important), pour off, and wash in aq. dest.

(3) Counterstain in either a 0.5 per cent. solution of Toluidin blue or a saturated solution of thionin in aq. dest. for one to two minutes.<sup>1</sup> Wash in aq. dest.

(4) Differentiate in a 0.5 per cent. solution of aurantia in

<sup>1</sup> I find less time than this sufficient. Moreover, in my hands the differentiation period is not during paragraph (4), but during subsequent treatment in the 96 per cent. alcohol. This is the best stain, other than iron hæmatoxylin, that I have used.

70 per cent. alcohol for from twenty to forty seconds, watching process under microscope.

(5) Wash in 96 per cent. alcohol, then absolute, xylol, and balsam.

The chromatin is blue, mitochondria are red, and the ground cytoplasm is golden-yellowish to green.

### 19. Remarks on Silver Nitrate Methods.

One of the oldest methods for the demonstration of cell-walls is the silver nitrate technique. Fresh tissue is treated with  $\text{AgNO}_3$  solution, and the salt is reduced in sunlight. This method is fairly capricious, and the preparations so produced are liable to after-blackening. The Golgi apparatus of Vertebrata especially can be demonstrated by the methods of Cajal and Golgi; these methods consist essentially in the fixation of a tissue in formalin followed by a bath of silver nitrate solution; the nitrate is reduced and then toned. Those who have not used these methods and whose knowledge of silver nitrate methods consists of an acquaintance with the old  $\text{AgNO}_3$  technique for cell-walls are liable to be led away wrongly by the view that Golgi and Cajal's methods are capricious and untrustworthy. I find, and several of my co-workers have found more recently, that these methods are remarkably uniform in their results provided proper care is taken. No histological method gives completely uniform results, but to identify the bodies which one sees in Cajal and Golgi preparations as artefacts is absurd. That this is so I hold for several reasons: Firstly, the methods are very uniform in result, and give the same results on many different sorts of animals; secondly, the methods of Cajal and Golgi demonstrate the bodies which are shown by  $\text{OsO}_4$  (Kopsch's method); thirdly, these silver methods impregnate bodies, which in the case of germ-cells may be seen in the living cell, or stained with intra-vitam stains. Lastly, these methods demonstrate bodies which can be, and have been, followed through the germ-cell cycle in all stages. These facts cannot



be refuted by critics of the silver nitrate methods, and are, I believe, conclusive.

## 20. The Golgi Apparatus Method of Golgi.

In my former paper (13) on technical methods I gave Cajal's Golgi apparatus technique. Another method, closely similar in some ways, is that of Golgi; both of these techniques consist in the fixation of the tissue in some fluid containing formalin, then immersion of the fixed material in  $\text{AgNO}_3$  for some time, and subsequent reduction of the silver salt in a fluid containing hydroquinone; in a later stage excessively reduced silver is toned in "hypo" mixture. Two main difficulties in such techniques are firstly to attain just the right length of time for soaking the material in the silver nitrate, and then secondly to reduce the silver just to the correct stage. After one or two trials subsequent attempts generally succeed.

A. Golgi first fixes six to twenty-four hours in the following mixture :

Formalin of 20 per cent. . . . .	30 c.c.
Saturated sol. arsenious acid . . . . .	30 c.c.
Alcohol of 97 per cent. . . . .	30 c.c.

b. Tissue transferred to 1 per cent.  $\text{AgNO}_3$  for from one hour to several days.

c. Reduced for two or three hours in mixture :

Hydroquinone . . . . .	20 gm.
Sodium sulphite . . . . .	1 gm.
Formalin . . . . .	20 c.c.
Aq. dest. . . . .	1000 c.c.

d. After this the pieces of tissue are washed about ten minutes in distilled water and then dehydrated as quickly as possible, and embedded in wax or celloidin and cut about  $6\mu$ .

e. Sections on slide are toned (differentiated) in following mixture :

- |                                   |             |
|-----------------------------------|-------------|
| (1) Sodium hyposulphite . . . . . | 30 gm.      |
| Ammonium sulphocyanate . . . . .  | 30 gm.      |
| Aq. dest. . . . .                 | 1000 c.c.   |
| (2) Gold chloride . . . . .       | 1 per cent. |

Mix equal parts of solution (1) and (2) just before use. Tone the sections till the cells look clear and the Golgi elements stand out. Wash in aq. dest., dehydrate, clear, mount in balsam (see Pappenheimer, 25).

As will be noted the fixative contains a good deal of alcohol which I consider bad technique. While Golgi's method may give fine result for mammalian material, there is no doubt that Cajal's fixative (4, 13) produces a better result for more delicate tissues.

Some of the material after fixation in Cajal or Golgi formalin mixture can be treated as for the formalin and iron-haematoxylin method on p. 286. I find such fixation to give very satisfactory results apart from the impregnation of the Golgi apparatus.

#### 21. Chromosome Work and Urea.

It has lately been claimed by Hance (16) that the addition of about  $\frac{1}{2}$  per cent. urea to fixatives of the Bouin or Flemming type prevent the clumping together of chromatin structures such as chromosomes or chromatin filaments, and enable the worker to count with ease such difficult chromosome plates as those of Vertebrata. My personal experience of such modified fixatives is at present not extensive enough to permit me to express an opinion. (For a reference to this see 'Journ. Roy. Micr. Soc.,' 1917, p. 347.)

#### 22. Vertebrate and Invertebrate Tissues.

It is very rare to find that one single method will produce the same good result in both Vertebrate and Invertebrate tissues. In the same way, methods which act satisfactorily with Amphibia will often give disappointing results with Mammalia. Osmic-chrome fixation will nearly always be found excellent for all classes of Invertebrata; Flemming-without-acetic acid can be highly recommended. For Amphibia the addition of some  $K_2Cr_2O_7$  to the Flemming is necessary before a correct fixation of the mitochondria is obtained; thus Champy's fluid was invented for Amphibia and gives very satisfactory results.

For mammalian tissues a preliminary fixation in osmic acid fixatives is not generally indicated; the tissues of mammals are far more "fatty" than those of Invertebrata or Amphibia, and one finds that the  $\text{OsO}_4$  becomes reduced very rapidly and penetration is very poor. For mammalian tissues formalin-chrome or formalin alone are indicated as a preliminary treatment at the least. Formalin does not destroy lipins, and by subsequently placing small pieces of formalin-fixed tissues in osmic acid, a fixation of lipins and fats is obtained; the same result may be got by fixing tissues in chrome salts and then transferring to osmic acid. It should be noted, however, that previous fixation in a chrome salt prevents the blackening of the Golgi apparatus; the formalin-osmic acid method (Sjövall) is indicated especially for mammalian tissues, when an impregnation of the Golgi apparatus is required by means of an osmic method, and the formalin silver nitrate techniques always give results of value.

### 23. Dark-ground Illumination and the Cell

#### Inclusions.

While it is true that very small micro-organisms can be conveniently seen with the well-known dark-ground illumination, the problem of studying the mitochondria and Golgi elements by this means is a difficult one. A spirochæte or bacillus floating in blood-plasma lies in a fluid substance of a different refractive index, while a mitochondrion or a Golgi rod is generally of nearly the same refractive index as the protoplasm in which it lies; in *Limnæa* eggs the mitochondria are easily seen with the ordinary microscope illumination because they are impregnated with a lipochrome, but under dark-ground illumination such granules are less easily seen. The Golgi (Nebenkern) elements in *Helix* spermatocytes are tolerably well seen in the fresh cell under ordinary direct illumination, because they differ slightly in refractive index from the protoplasm, but in the nerve-cell the Golgi elements are to my knowledge imperceptible under the same conditions; yet both Kopsch and Golgi or Cajal techniques demonstrate

a Golgi apparatus in both *Helix* spermatocyte and nerve-cell. In the former case direct observation reveals that such staining techniques impregnate bodies which can be seen *intra vitam*.

With the most critical conditions the mitochondria and Golgi elements can be studied in the live cell using dark-ground illumination, but this method will not replace fixation and staining techniques, and at best merely shows granules as pale rings of light.

#### 24. The Polariscope Microscope as an Adjunct to Cytological Research.

Some help in distinguishing between or merely detecting substances in the cells of tissues may be gained by the use of a polariscope microscope. The theory of this instrument can be read elsewhere (31), but it is well known that those substances, which are singly refracting, are quite dark between crossed nicols and remain so during a complete rotation of the stage, and are called isotropic, while those substances which show themselves as points of light between crossed nicols and are alternately dark and illuminated when the stage is rotated are known as anisotropic. True neutral animal fat is isotropic, while certain lipins are anisotropic (7). The development and use of this method of examining zoological material has as yet been inextensive, and it is possible that such a method of examining fresh eggs and embryos may yield some interesting facts when used in conjunction with other cytological methods.

Polarised light will not produce a result in every case; one of my most perfect Kopsch ( $\text{OsO}_4$ ) slides of the *Limnæa* ovotestis, which under the ordinary microscope revealed mitochondria, Golgi apparatus and yolk-discs very clearly, was quite isotropic. This may be due to the  $\text{OsO}_4$  altering the optical properties of the intracellular materials. Ovaries and testes may be teased out fresh in their own juice and examined *intra vitam*. This gives the best result, and possibly the only reliable one.

## 25. General Plan for Cytological Research.

The zoologist meets with the following bodies in the cell Nucleus, and in the cytoplasm, Golgi apparatus, mitochondria, fat, yolk, and glycogen. He may also find pigment-granules.

(1) Some of the material to be worked on must be fixed in chrome-osmium, of which fluids Flemming-without-acetic acid, Altmann, and Champy may be tried. In testes, ovaries and in eggs this is certain to preserve nearly all the cell elements, i. e. mitochondria, Golgi apparatus, fat and yolk. It is doubtful whether glycogen is preserved. On staining, the mitochondria will go black in iron-hæmatoxylin, or red in acid fuchsin (Altmann, Champy-Kull, or Bensley). The Golgi apparatus may stain in the case of the male germ-cells, but not generally in the egg or embryo; yolk is generally yellowish green-brown, or more rarely black, and remains so even after the Altmann stain. On staining in iron-hæmatoxylin yolk may or may not turn black; in the frog this stain turns yolk black, in the snail it does not do so. In these preparations fat goes black also; but fat may easily be distinguished from other elements by mounting some sections unstained, or simply by staining in acid fuchsin (Altmann). In these preparations what is black is almost certain to be fat or contain fat, because chrome-osmium alone, so far as my experience goes, never turns the Golgi elements or mitochondria black. By this chrome-osmium fixation one gets a good preliminary survey of the mitochondria and other cell elements.

(2) The next stage may be taken as follows: Some tissue is fixed about two days in 5-10 per cent. formalin, which should be carefully neutralised in magnesium carbonate. Dehydration and clearing is carried out in alcohol and xylol respectively. Staining should be done in iron-hæmatoxylin. The result of this technique is to get rid of fat, but to stain mitochondria densely black. In eggs and embryos the Golgi apparatus generally does not show, but it may do so in the male germ-cells. The effect of fixing material in chromic

acid or bichromate of potassium with formalin is nearly the same as with formalin alone; none of these reagents has the power of making fat insoluble in alcohol + xylol, but all have the power of making mitochondria insoluble in the same reagents. In formalin yolk is preserved, and may go black in iron-hæmatoxylin, but I do not know of its going red in acid fuchsin. Fat is lost.

(3) The next step is to attack the problem of the Golgi apparatus. Two methods should be tried.

(A) Kopsch's osmium tetroxide methods, in which small pieces of tissue are treated in the osmic fluid (2 per cent.) for from one to fourteen days, then sectioned and mounted unstained, or stained in acid fuchsin (Altmann). The Golgi apparatus will go black; by this method fat also will go black, and yolk may do so. The mitochondria almost always remain yellowish or unstained by the osmic fluid, and subsequent treatment of the material in acid fuchsin will turn these granules red. The Golgi apparatus remains black (see also various Kopsch modifications, p. 284). Such preparations can with advantage be compared and contrasted with those made by chrome-osmium methods as in paragraph (1).

(B) The other method which should be used is that of Golgi or Cajal. In my previous paper on this subject (13) I gave the method of Cajal, and I have described that of Golgi here (p. 292). Cajal's method differs from Golgi's in one very important respect, i. e. that the latter's fixing fluid contains a good deal of alcohol. Both Golgi and Cajal's silver methods for the "apparato interno reticolare" are progressive, and in one block of tissue one may find regions where no "apparato" is impregnated, regions where only the Golgi apparatus is impregnated, and regions where both Golgi elements and mitochondria are black. In typical cases, however, the latter go yellowish to brown. Material fixed in Cajal or Golgi fixative may be stained in iron-hæmatoxylin.

(4) As a general rule it is advisable to fix and stain for glycogen. There are two methods—the iodine (13) and Best's carmine. Details of these will be found either in Bolles



Lee's 'Microtometist's Vade-mecum' or Mallory and Wright's 'Pathological Technique.'

(5) Some material should be fixed in a fluid such as that of Bonin, Carnoy, Gilson-Petrunkewitsch, Zenker or Flemming-with-acetic acid, and stained in Ehrlich's hæmatoxylin and eosin, or in iron-hæmatoxylin and orange G. These preparations are useful for details of spindle-fibres, centrosome, and especially for ascertaining oxyphility and basophilicity of ground cytoplasm, or of nuclear bodies. Such preparations are also a *sine quâ non* where a study of chromosomes is to be carried out, because chrome-osmium and other mitochondrial fixatives not containing alcohol and acetic acid do not generally give a sharp picture of chromatinic structures. For ascertaining oxyphility, amphophility or basophilicity staining in Ehrlich's hæmatoxylin and eosin or Biebrich scarlet gives good result. This method was perfected by the late Dr. S. G. Scott of the Histology Laboratory, Oxford. Its main advantage is that it gives a correct histological picture of the degree of oxyphility or basophilicity of any cell body or of the ground cytoplasm, without over-staining and washing out, which introduces complications.

(A) Bring sections to 90 per cent. alcohol and wipe slide dry except around sections. Add just enough drops of Ehrlich's hæmatoxylin to cover sections. Leave slide on bench for ten minutes.

(B) Drain away hæmatoxylin, and wash off every trace of latter with 90 per cent. alcohol, and then with aq. dest.

(c) Blue the sections in the following standard tap-water substitute:

KHCO <sub>3</sub>	.	.	.	.	.	.	2	gram.
MgSO <sub>4</sub> + 7H <sub>2</sub> O.	.	.	.	.	.	.	20	gram.
Aq. dest.	.	.	.	.	.	.	1000	c.c.

Add lump of thymol.

Leave some drops of this mixture on slide for three to four minutes, and then wash in distilled water thoroughly.

(D) Wipe around sections, and add some drops of weak eosin (1 in 1000 in aq. dest.); leave ten minutes till pink or



reddish (control under microscope). (Scott uses particularly Biebrich scarlet instead of eosin, as the former is less diffuse.) Drain off stain, quickly wash in aq. dest.

(E) Blot, dip into absolute alcohol and quickly transfer to xylol. Mount in balsam.

(6) During the preparation of these sections it is advisable to examine both fresh and intra-vitam stained teased preparations. The animal is killed by a blow on the head, or if an invertebrate, by cutting off its head, the gonads or other organs are rapidly removed and pieces are teased in the animal's own juice (lymph), or less preferably in normal saline, and examined as soon as possible. After examination of fresh cells (or eggs) the teased material may be examined as follows:

(A) Freshly teased cells to which Janus green has been added (Janus green in normal saline solution, 1 in 20,000 to 1 in 30,000). (Results depend greatly on the particular specimen of dye.)

(B) Ditto in neutral red, 1 in 10,000 to 1 in 30,000.

(C) Ditto in  $\text{OsO}_4$  of 1 per cent., and in weak permanganate of potassium. (See also 13.)

(7) In addition the dark-ground illumination and the polariscope should be tried, but it should be noted that neither of these contrivances at present provide the researcher with extensive lines of evidence.

## 26. CONCLUSIONS.

In these notes I have indicated the general trend of the more modern cytology. In the previous paper I gave tables and figures which should enable workers not familiar with the cytoplasmic inclusions to identify any given body. The last few years have seen a great advance in technique, and workers should now be able to classify and follow separately any given substance or material in the egg or embryo, as well as in the gland.

It should not, however, be considered that the problem of the cell organs and granules is solved—far from it—for there

are (especially in gland-cells and protozoa (10)) enigmatic bodies, possibly of an obscure metabolic nature, whose true identity has not been established. Nevertheless, I consider that such rapid advances have been made that there are now few cell bodies which the zoologist cannot easily identify and follow during organogeny and gametogenesis.

What is now wanted, and would be of incalculable value, would be some method which would reveal chromatin in all its stages, and which would enable us to follow out the interchanges between nucleus and cytoplasm.

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June 20th, 1919.

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